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NOTES ON TWO "ATOXIC" STRAINS OF DIPHTHERIA BACILLI.*

HERBERT R. BROWN, M.D., AND THEOBALD SMITH, M.D.

(*From the Antitoxin and Vaccine Laboratory, Massachusetts State Board of Health and the Department of Comparative Pathology, Harvard Medical School.*)

The existence on the mucous membranes of convalescent, recovered, and well persons of bacilli biologically indistinguishable from genuine diphtheria bacilli, but incapable of toxin production, has been referred to from time to time in bacteriological writings. These types are readily differentiated from the Hofmann or pseudo-diphtheria bacillus by their behavior in dextrose bouillon in which a certain amount of acid is produced, by their morphology, and by the presence of the Neisser granules.

Culture No. 49, obtained through Dr. Denny from the Brookline Board of Health in February, 1900, had been isolated from the throat of a healthy adult. The culture was brought to the laboratory because no pathological effects could be obtained on guinea-pigs even with large doses. The laboratory diagnostician regarded the morphology as that of the virulent type. Neisser's granules were present. Five and one-half per cent of a normal acid was produced in dextrose bouillon. On Löffler's serum when examined again four months later, the bacilli were short, plump, usually in pairs without Neisser granules. After a further period of three and one-half months the culture, when twenty-four hours old, presented a somewhat different appearance. The bacilli were now long and slender, with club-shaped or swollen extremities with more or less segmentation of the body into short blocks. Neisser's stain failed to show any granules. The capacity to produce toxin was tested on standard bouillon which was used for the

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production of diphtheria toxin on a large scale. One cubic centimeter of a forty-eight-hour culture, unfiltered, failed to produce a local induration. One cubic centimeter of a nine-day culture unfiltered produced a swelling in the subcutis about two cubic centimeters in diameter. This subsided rapidly within two days, when it was about half its original size. In eight days the guinea-pig had gained twenty grams; in twelve days no trace of the induration was palpable.

After artificial cultivation on Löffler's serum from February to October, 1900, a second guinea-pig received subcutaneously two cubic centimeters of a ten-day bouillon culture. A pea-sized nodule formed at site of inoculation which disappeared later. The weight rose thirty-five grams in the six days following the injection.

Culture No. 50 was obtained from the throat of a boy twelve years of age five and one-third months after the first symptoms of clinical diphtheria. Positive cultures were obtained every week or two after the subsidence of the disease. Culture No. 50, isolated about May 16, was followed by other positive cultures until June 21, when cocci only were reported. The family physician who had charge of the case regarded it as an attack of genuine diphtheria followed in two weeks by reappearance of the throat lesions. All symptoms were over in four weeks.

Examined after artificial cultivation for four months the bacilli on Löffler's serum were short without club-shaped ends. They contained deeply-stained segments with attenuated, feebly-stained extremities. Neisser's granules were present. Three and one-half months later the bacilli in a twenty-four-hour culture were more slender. Neisser's granules one to three in a cell, chiefly terminal. It is probable that these changes in form may come under the phenomenon of mutation as recently described by Baerthlein.[1] Acid reaction in dextrose bouillon equivalent to 5.65 per cent of a normal acid.

In order to place this culture in the most favorable condition for toxin production it was grown in standard bouillon through nineteen passages from May 22 to July 27, 1900. At this time a thin membrane formed regularly. The bouillon became clear by rapid sedimentation. Two flasks were inoculated from the nineteenth transfer; (*a*), one of fermented bouillon plus two per cent peptone but without sugar, and (*b*), the same bouillon plus .1 per cent dextrose. The final reaction at the end of eleven days was .05 and .25 per cent alkaline respectively to phenolphthalein.

Of each of these cultures one cubic centimeter (shaken up into a turbid mass) was injected into a guinea-pig subcutaneously. Neither guinea-pig showed any lesions subsequently. In the one receiving the bouillon without dextrose the weight fell one gram. In the other it rose twenty-six grams in five days. These two cultures have remained in the collection of the Department of Comparative Pathology, Harvard Medical School, and recently it seemed advisable to make a few additional tests to determine the relationships of these two strains to the toxin-producing strains. At the same time the two cultures were tested on various carbohydrates and alcohols to determine anew their relation to true toxin producers. The following table gives the results. The fermentable substances were added to fermented bouillon to make a one-half per cent solution. The cultures were incubated at 37° C. and removed and titrated after eight days. These tests were made December, 1913. The figures indicate the acidity or alkalinity in per cent of a normal solution, n/20 solutions being used with phenolphthalein as indicator.

TABLE I.

One-half Per Cent Sugars, etc.	Diphtheria No. 49.	Diphtheria No. 50.	
Fermented bouillon (sterile control) . .	. . . .	. . . .	0.45 acid.
“ “	1.20 alkaline.	0.00	
“ “ plus dextrose . . .	**4.95** acid.	**3.30** acid.	
“ “ “ galactose . . .	**3.65** “	**4.05** “	
“ “ “ levulose . . .	**3.00** “	**4.25** “	
“ “ “ maltose . . .	0.60 “	1.50 “	
“ “ “ dextrine . . .	0.15 alkaline.	0.40 “	
“ “ “ lactose	0.70 “	0.00 “	
“ “ “ saccharose . .	0.35 “	0.00 “	
“ “ “ raffinose . . .	0.20 “	0.20 alkaline.	
“ “ “ mannite . . .	0.60 “	0.00 “	
“ “ “ inulin	0.36 “	0.00 “	

The table indicates that both cultures produced acids in dextrose, galactose, and levulose and not in the other substances. The only doubtful result is the figure for maltose bouillon under Culture No. 50. In this tube there was a slight rise in the acid reaction, from .45 to 1.50 per cent.

It was next determined to examine again the production of toxin, its immunizing effect on guinea-pigs, and its relation to diphtheria antitoxin.

Culture No. 49.—The later studies of Culture No. 49 showed that local lesions and death may be produced by very large doses injected subcutaneously. The lesions were, in gross appearance, the same as those produced by the filtrates of the true diphtheria bacilli. Stored filtrates maintained a certain degree of toxicity for nearly two years as the following table shows:

TABLE II.

Guinea-Pig No.	Weight, Grams.	Dose, cc.	Date of Injection.	Result.
505	285	5	Sept. 23, 1911.	Death in $2\frac{1}{2}$ to $2\frac{3}{4}$ days. Heavy subcutaneous edema. Thorax full of serum.
533	283	5	" 30, "	Death in 3 days. Hemorrhagic edema in subcutis.
1605	266	2	Aug. 13, 1913.	Slough and ulcer locally.
1613	268	5	" 13 "	Large slough. Great loss in weight. Paralysis.

To determine the immunizing effect of bouillon filtrates, young female guinea-pigs were immunized actively, then put to breeding and their offspring tested for any passive immunity they may have acquired according to methods already published.[2, 3, 4, 5]

The cultures were prepared at first in test-tubes one inch in diameter and inclined so as to expose a large area of liquid to the air. Later Fernbach flasks were employed with the result of obtaining a more toxic fluid. The bouillon was prepared from fresh veal fermented with B. coli, to which, before final inoculation with the throat bacilli, .2 per cent dextrose was added. Two guinea-pigs were immunized with bouillon filtrates, but unfortunately one died at the end of the treatment because the toxin used was much stronger than previous doses. At the same time one female guinea-pig was treated with increasing doses of living bouillon cultures incubated from one to four days.

The treatment of these animals and the results obtained on testing their offspring are given in Table III. It will be noted that no increase in resistance above the normal was evident. The active immunity produced in the mothers, if any, was so slight that none was transmitted to the offspring.

TABLE III.

Living culture No. 49.

Mother.					Offspring.					
Date.	Dose of Bouillon Culture Injected and Hours Incubation.	Result.	Loss in Weight in Grams.	Days Between Injections.	Period Between Last Injection of Mother and Birth of Litter.	Age in Days.	Weight in Grams.	Dose of Toxin Injected + One Antitoxin Unit.	Result.	Normal L + Dose.
		No. 405.								
1911. Aug. 1 .	1 cc. 72 hrs.	Subcut. nodule.	O		120 days.	19	*First* 268	*litter.* .18	Dies 2⅞ days.	.18
' 12 .	1 ' 72	' ' 1 x ¼ in.	O	11						
" 23 .	1 ' 24 "	" " ½ x ⅛ in.	6	11						
Sept. 2 .	2 " 24	Swelling 1 x ½ in.	27	10	206 '	24	*Second* 294	*litter.* .183	' 3½ '	.183
9 .	3 " 24 '	Mod. edema 1¼ in diam.	10	7						
16 .	5 ' 96	' 1¼ " "	7	7						
Oct. 19 . .	3 ' 24 "	Sl. " 2 x 1 in.	45	33						
Nov. 3† . .	2 48 '	Swelling ½ in. diam.	. . . .	15						
Dec. 9 . . .	3 24	' 1 x ¾ in.	. . . .	37						
1912. Jan. 12 . .	4 ' 48 '	Induration 2 x 2 x ¼ in.	. . . .	34						
Feb. 3 . .	5 ' 24	Swelling 1 x ½ x ¼ in.	48	22						
Total .	30 cc.									

Bouillon filtrate No. 49.

No. 393.										
1911. Oct. 11 . .	.50 cc.	Mod. edema.	75		104 days.	27	*First* 282	*litter.* .18	Dies 2½ days.	.18
Nov. 3 . .	.75 “	“	2	23	104 ‘	35	392	.18	‘ 2½ ‘	.18
Dec. 9 . .	.90 “	‘	. .	36						
1912. Jan. 12 . . .	1.00 ‘	‘ “	. . . . .	34	174 “	33	*Second* 300	*litter.* .18	‘ 3 ‘	.18
Feb. 3 . . .	1.25 “	Ulcer ⅛ x ¼ in. Sl. paralysis.	41	22						
Total .	4.40 cc.									
No. 395.										
1911. Aug. 16 .	1 cc.	No lesion.								
“ 23 .	1 “	“ ‘								
“ 26 .	2 “	“ “								
Sept. 2 .	5 “	Swelling 1 x 1½ in.								
“ 9 . .	5 “	Sl. edema.								
“ 16 . .	5 ‘	“ ‘								
‘ 30 . .	5 ‘	Heavy edema.								
Oct. 4 . .	5 ‘	Dies in 3 days.*								
Total .	29 cc.									

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* The toxin injected was made in a Fernbach flask. The other doses were quantities from different lots of test-tube cultures.

† Culture on Löffler's serum.

The relation of the toxin produced by No. 49 to true diphtheria toxin was shown by mixing the toxin with standard serum and injecting the mixture.

TABLE IV.

Guinea-pig No.	Weight, gms.	Toxin, cc.	Standard Serum, Units.	Result.
1656	250	5	½	No lesion.
1703	257	5	1/20	" "
1929	257	5	. . . .	Large slough, ulcer, paralysis.

This table shows that one-twentieth antitoxic unit completely neutralizes the pathological action of five cubic centimeters of the culture filtrate.

Culture No. 50. — The unfiltered fluid from cultures even in doses of five cubic centimeters produced only a moderate to heavy edema, frequently leading to the formation of small subcutaneous nodules but not to necrosis. The injection of the filtrate in doses of five cubic centimeters produced edema and induration which persisted in the form of small nodules for a time. More rarely, superficial necrosis or scab-formation followed the edema. The loss in weight was nominal as Table V. indicates. The more severe effect of the filtrate may be referred to the longer incubation of the culture.

The few offspring that were available for subsequent immunity tests possessed no greater than normal resistance to the L + dose of the toxin-antitoxin mixtures as shown in the following table:

TABLE V.

Living culture No. 50.

Mother.					Offspring.					
Date.	Dose of Bouillon Culture Injected and Hours Incubation.	Result.	Loss in Weight in Grams.	Days Between Injections.	Period Between Last Injection of Mother and Birth of Litter.	Age in Days.	Weight in Grams.	Dose of Toxin Injected + One Antitoxin Unit.	Result.	Normal L + Dose.
No. 406.										
1911. Aug. 1 . .	1 cc. 72 hrs.	Subcut. nodule.	O		113 days.	26	*First litter.* 270	.18	Dies 3⅛ days.	.18
" 12 . .	1 72 "	"	. . . .	11						
" 23 . .	1 24	"	6	12						
Sept. 2 . .	2 24	Swelling 1½ x 1½ in.	21	10	180 "	27	*Second litter.* 243	.18	" 3½ ± "	.18
" 9 . .	3 24 "	Slight edema ¾ in.	9	7						
16 . .	5 96 "	Edema 2 x 1½ in.	16	7						
Oct. 19 . .	5 " 24 "	3 x 2 in.	52	33						
Nov. 3 . . .	2 " 24 "	Swelling ½ x ¼ in.	. . . . . .	15						
Dec. 9 . . .	5 " 24	Induration 1 x 1 x ¼ in.	. . . .	37						
1912. Jan. 12 . .	5 48	" 1 x 1 x ½ in.	. . . . . .	34						
Feb. 3 . . .	5 " 24	Swelling 1 x ¾ x ¼ in.	28	22						
Total .	35 cc.									

TABLE V. — *Continued.*

Bouillon filtrate No. 50.

Mother.					Offspring.					
Date.	Dose of Bouillon Filtrate Injected.	Result.	Loss in Weight in Grams.	Days Between Injections.	Period Between Last Injection of Mother and Birth of Litter.	Age in Days.	Weight in Grams.	Dose of Toxin Injected + One Antitoxin Unit.	Result.	Normal L + Dose.
		No. 407.								
1911. Aug. 16	1 cc.	No lesion.	0		117 days.	22	*First litter.* 281	.18	Dies 3⅛ days.	.18
" 23	1	" "	0	7			*Second litter.* All born dead.			
" 26	2 "		0	3						
Sept. 2	5 "	Swelling 2 x 1 in.	10	6						
9	5 "	Mod. edema.	0	7						
" 16	5	Swelling 1 x 1 in.	13	7						
" 30	5 " *	Mod. edema.	7	14						
Oct. 19	5 *	Heavy "	24	19						
Nov. 3	5 " *	Mod. "	. . . . .	15						
Dec. 9	5 " *	Nodule ¾ x ½ in.	. . . .	36						
1912. Jan. 4	5 " *	Swelling 1 x 1 in.	. . . .	26						
Feb. 3	5 " *	1 x ½ in.	23	30						

* Bouillon filtrate injected was from a lot made in a Fernbach flask. The preceding injections were made with filtrates of test-tube cultures.

In order to test the effect of standard diphtheria antitoxin upon any toxic action that might be exerted by filtrates of No. 50, fresh filtrates were prepared from cultures in fermented bouillon to which various amounts of dextrose had been added. Only slight transient edema was produced in guinea-pigs with five cubic centimeters of filtrates from seven to fifteen day cultures. Even when one-half unit of antitoxin was injected with the filtrates, the same slight edema was produced. This result does not necessarily prove that Culture No. 50 is not a true diphtheria strain, for the slight edema may be due to constituents of the diphtheria bacillus other than the true toxin towards which the antitoxin may have no neutralizing power.

TABLE VI.

Filtrates of Culture No. 50.

Guinea-pig No.	Fermented Bouillon Plus	Dose of Toxin Injected.	Amount of Antitoxin Added.	Initial Weight in Grams.	Weight in Grams on Fourth Day After Injection.	Lesion Produced.
1988 . . .	.05 per cent dextrose.	5 cc.	0	248	240	Edema one inch diameter on first day only.
1989 . . .	.10 per cent dextrose.	5 "	0	249	266	Trace edema on first day.
1985 . . .	.20 per cent dextrose.	5 "	0	250	258	No lesion.
1973 . . .	.05 per cent dextrose.	5 "	0	248	263	Very slight edema.
1970 . .	.05 per cent dextrose.	5 "	1/2 unit.	238	242	" " "

We may summarize the results of our study of these two strains as follows: Both are morphologically and culturally true diphtheria bacilli. They are not pseudo-diphtheria strains, for the latter are strictly aërobic, non-fermenting organisms. We do not agree with Trautmann and Gaehtgens [6] or Bernhardt and Paneth,[7] who assume that one type may pass into the other under certain conditions.

Strain No. 49 is feebly toxic, a minimum fatal dose being contained in five cubic centimeters or more of the filtrate obtained from cultures placed under most favorable conditions for toxin production. Number 50 is practically atoxic. The immunization of female guinea-pigs with either strain produced no appreciable effect on the resistance of the offspring to genuine diphtheria toxin.

The toxic action of the filtrate from Culture No. 49 was completely neutralized with diphtheria antitoxin, while that of No. 50 was so feeble that no inference can be drawn from the neutralization test which was apparently negative.

The main lesson to be drawn from these tests is that diphtheria bacilli should not be classed as atoxic until comparatively large doses of culture filtrates have been injected into guinea-pigs without producing local lesions. When such lesions as edema or induration occur, antitoxin should be tried as a neutralizing agent to determine the specificity or non-specificity of the substance causing the local lesion. In our tests the repeated injection of filtrates and bacilli in very large doses produced no appreciable active immunity, for the offspring possessed no more than normal resistance.

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